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Cloning of Noncultivatable Human Rotavirus by Single Primer Amplification

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A novel, sequence-independent strategy has been developed for the amplification of full-length cDNA copies of the genes of double-stranded RNA (dsRNA) viruses. Using human (Bristol) group C rotavirus as an example, a single amino-linked modified oligonucleotide (primer 1) was ligated to either end of each dsRNA genome segment by using T4 RNA ligase. Following reverse transcription, annealing, and repair of cDNA strands, amplification of the viral dsRNA genome was accomplished by polymerase chain reaction using a single complementary oligonucleotide (primer 2). Northern (RNA) hybridization of cDNA to virus dsRNA indicated that it was possible to generate cDNA representing the complete genome from very small clinical samples. This technique was used to determine the complete nucleotide sequence (728 bp) and coding assignment of gene 10, which revealed an open reading frame of 212 amino acids with limited homology to NS26 from human group A rotavirus. In contrast to previous tailing methods, the addition of one defined primer allowed unequivocal identification of terminal nucleotides and should be generally applicable to viruses with segmented dsRNA genomes and especially for analysis of clinical samples, for which very limited quantities of biological material are available.

Rotaviruses are major etiological agents of acute infantile gastroenteritis. Most infections are caused by group A rotaviruses and occur in children under 5 years of age. An important exception to this was a vast outbreak of adult diarrhea in China, first reported in 1983 (15), which was caused by the adult diarrhea virus (ADRV), a group B rotavirus (14). More recently, several accounts of small diarrheal outbreaks in infants and young children caused by atypical group C rotaviruses have been reported in England (6, 7). On the basis of the relative electrophoretic mobilities of their double-stranded RNA (dsRNA) genome segments, lack of cross-hybridization, fingerprinting studies, and serological differences, seven groups of rotaviruses (A to G) have been identified (for a review, see reference 21), although only three of these groups (A to C) have been shown to infect humans (5).

Rapid progress in characterizing group A rotaviruses followed the discovery that most animal isolates could be propagated by incorporation of trypsin into the tissue culture medium (1). The first group A human rotavirus was adapted to growth in tissue culture in 1980 (24). As an alternate approach, genes from group A isolates were recovered by gene rescue techniques using reassortment with tissue culture-adapted strains (13, 18). Unfortunately, no reports of successful reassortment between different rotavirus groups have been made. Although the Cowden strain of porcine group C rotavirus has been adapted to tissue culture (22), no human group B or group C rotaviruses have been adapted to growth in tissue culture, and there have been no reports of successful gene rescue by reassortment of these noncultivatable human rotaviruses.

Inability to propagate the atypical human rotaviruses in vitro has made it very difficult to prepare materials such as antibodies or hybridization probes for diagnostic reagents. Molecular cloning technology has provided an alternative approach to obtaining these materials. Original molecular cloning of rotavirus genes traditionally relied on the availability of large amounts of dsRNA in the starting material, a situation which is often difficult with clinical samples. Recently, two genes from the Cowden strain of group C porcine rotavirus purified from the feces of an experimentally infected pig have been cloned and sequenced (4, 20). Availability of this information has made it possible for primers to be designed to allow specific polymerase chain reaction (PCR) amplification of equivalent genes from noncultivatable group C human isolates (11, 20).

Clearly, a procedure was required to facilitate the molecular cloning of noncultivatable human rotavirus genomes directly from small clinical samples. This method should be applicable without prior knowledge of any sequence information and should generate full-length cDNA clones of each genome segment, allowing the unequivocal delineation of the 5′- and 3′-terminal gene sequences.

We report the development of such a novel technique for amplifying dsRNA genome segments that uses a single amino-linked oligonucleotide primer. We have applied this method to amplify and clone the genome of a human group C rotavirus from a family outbreak of gastroenteritis in Bristol, United Kingdom, which resulted in the death of an infant (7). As an example of the cloning procedure, we present the complete nucleotide sequence and coding assignment of gene 10.

Preparation of rotavirus dsRNA. The human group C rotavirus used in this study has been described previously (7). Samples were originally noted as non-group A because of the lack of reactivity with the standard group A enzyme-linked immunosorbent assay, but they exhibited classical rotavirus morphology on electron microscopic examination (Fig. 1A). This isolate was proven to be a group C rotavirus because of sequence similarity of its major inner capsid gene with the prototype Cowden strain of porcine group C rotavirus (11). The dsRNA profile of this human isolate is shown in Fig. 1B.

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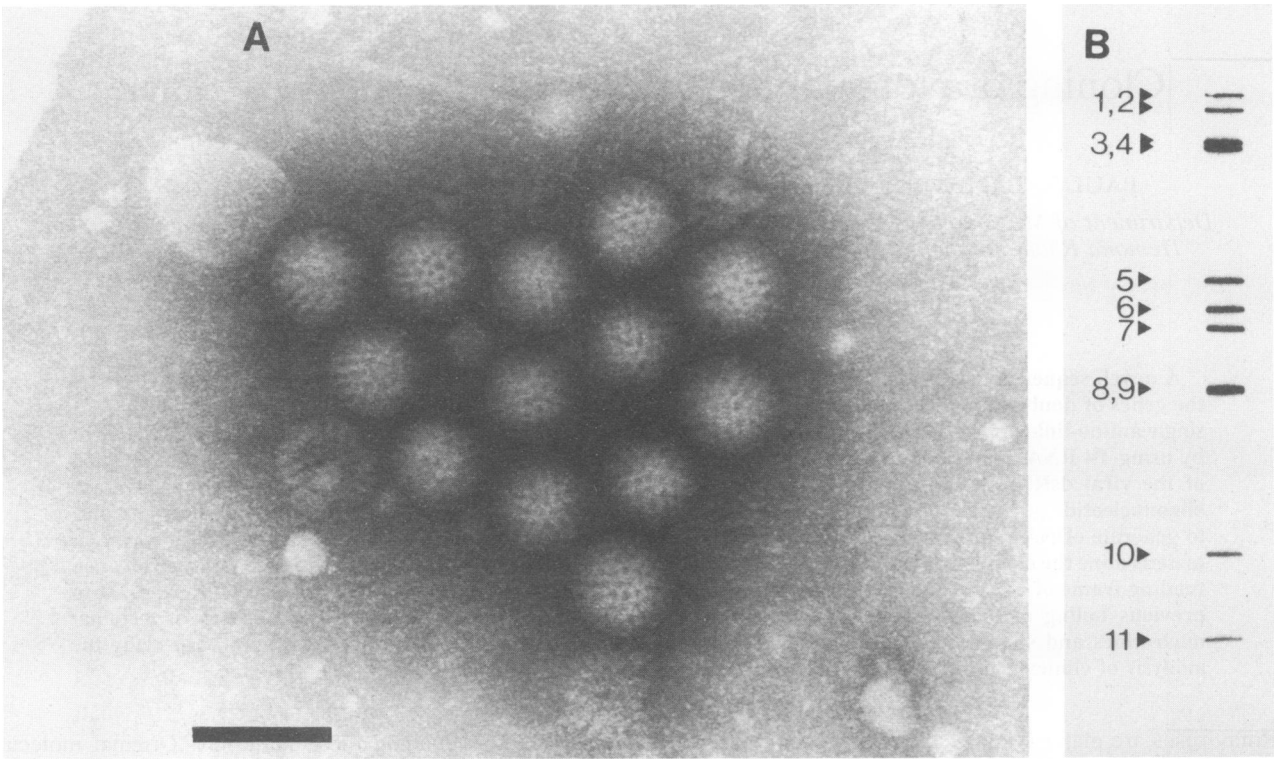

FIG. 1. (A) Electron micrograph of human (Bristol) group C rotavirus particles from the specimen used in this study. Rotaviruses were negatively stained for 2 min in 0.5% phosphotungstic acid (pH 6.0) and visualized with a Hitachi H7000 transmission electron microscope. The scale bar represents 100 nm. (B) Autoradiograph of a [^{32}P]pCp-labelled polyacrylamide gel profile of human (Bristol) group C rotavirus dsRNA prepared as described in the text and used in this study. Gene segments are marked on the left.

For extraction, genomic dsRNAs from human group C rotavirus fecal samples were prepared as 75% suspensions in Dulbecco's minimal essential medium (Life Technologies). Aliquots of the suspension were treated with RNase T_1 (100 U; BCL) at 55°C for 15 min prior to extraction with an equal volume of RNAzol B (Biogenesis Ltd.). Rotavirus genomic dsRNA was purified from the RNAzol B aqueous phase by using silica particles (Geneclean II) as previously described (2, 25). It was possible to specifically purify human group C rotavirus dsRNA from very small amounts of fecal samples. As little as 20 μl of liquid stool was enough material to yield clonable quantities of dsRNA, as judged by silver staining. Previous work (25) has shown that a single silver-stained rotavirus genomic profile in a fecal specimen represents approximately 8×10^9 rotavirus particles. From this figure, we estimate that a silver-stained rotavirus genomic profile represented 20 to 100 ng of dsRNA.

Sequence-independent PCR amplification of the group C rotavirus genome. Oligonucleotides for use as PCR and DNA sequencing primers were synthesized by using β-cyanoethyl phosphoramidite chemistry on a model 381A automated DNA synthesizer (Applied Biosystems Inc.). To prevent concatenation of primer 1 in subsequent dsRNA/DNA ligation reactions, a terminal 3′-NH_2 blocking group was incorporated, using 3′-Amine-ON CPG (Clontech Laboratories, Palo Alto, Calif.). Oligodeoxyribonucleotide primer 1 (5′-CCCGTCGACGAATTCTTT-3′-NH_2) (200 ng) was phosphorylated with 50 μCi of [γ-^{32}P]ATP and 10 U of T4 polynucleotide kinase at 37°C for 1 h in buffer containing 70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 5 mM dithiothreitol as described by Sambrook et al. (23). After 1 h, the reaction was chased for a further 1 h by addition of ATP to a final concentration of 10 mM. Phosphorylated oligonucleotides were purified from the kinase reaction components prior to DNA/dsRNA ligation by adsorption chromatography on a NENsorb column (NEN) according to the manufacturer's instructions.

The first step of the amplification process was to ligate 3′-amino-blocked oligodeoxyribonucleotides to both 3′ ends of the dsRNA genome segments. This was achieved by using the DNA/RNA ligation properties of T4 RNA ligase and approximately 20 to 100 ng of group C rotavirus dsRNA. Group C rotavirus genomic dsRNA marker profiles were prepared by labelling dsRNA at the 3′ termini, using cytidine 3′,5′-[5′-^{32}P]bisphosphate (pCp; Amersham) and T4 RNA ligase (Life Technologies) according to the manufacturer's instructions. For ligation of oligodeoxynucleotide primer 1 to dsRNA, the incubation time at 4°C was extended from 30 min to 16 h. Unligated primer 1 molecules were removed by spin column chromatography on Sephacryl S-400 (Promega). The successful addition of radiolabelled oligodeoxyribonucleotide to purified group C rotavirus dsRNA in the ligation reactions was monitored by agarose gel electrophoresis of spin column eluates (Fig. 2). Samples were heated at 65°C for 2 min prior to gel loading to ensure that the oligonucleotides had ligated to the dsRNA. This analysis revealed some loss in recovery of the smallest genome segments 10 and 11 after spin column chromatography on Sephacryl S-400.

Primer 1-tailed rotavirus genomic dsRNA was denatured

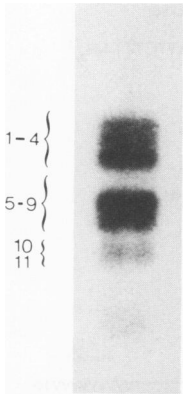

FIG. 2. Tailing of rotavirus dsRNA with primer 1. Rotavirus dsRNA was tailed with primer 1, using T4 RNA ligase. An aliquot (3 μl) of the reaction mixture was analyzed (after spin column chromatography) by electrophoresis in a 1% standard agarose gel at 90 V for 90 min (23). The genome segments are marked by numbered braces and were verified by purification of bands from the gel and reelectrophoresis on polyacrylamide gels.

by heating to 90°C for 5 min in aqueous dimethyl sulfoxide (18% [vol/vol], final concentration) in the presence of 90 ng of primer 2 (5′-AAAGAATTCGTCGACGGG complementary to primer 1) and cooled rapidly on ice. The denatured RNA was converted to cDNA with RNase H^- reverse transcriptase (Superscript) in a reaction mixture containing the following in a final volume of 50 μl: dimethyl sulfoxide-denatured RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each deoxynucleoside triphosphate (dNTP), 100 μg of bovine serum albumin per ml, 40 U of RNasin (Promega), 50 μCi of [α-^{32}P]dCTP, and 500 U of Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies). After incubation at 42°C for 60 min, the reaction was stopped by addition of EDTA (10 mM, final concentration). The remaining RNA was hydrolyzed by addition of 5 μl of 1 M NaOH and heating to 65°C for 60 min, followed by cooling to room temperature and neutralizing with 5 μl of 1 M HCl plus 5 μl of 1 M Tris-HCl (pH 7.5). The resulting cDNA representing the plus and minus strands of the genomic RNA was annealed at 65°C for 16 h prior to purification by spin column chromatography on Sephacryl S-400. Partial duplexes formed by annealed strands were repaired by using *Taq* DNA polymerase (Promega) prior to amplification by PCR using a single primer (primer 2).

Careful choice of PCR conditions allowed amplification of cDNA in the size range representing the complete group C rotavirus genome. Rotavirus cDNA was incubated with *Taq* DNA polymerase in a reaction mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each dNTP, and 2.5 U of *Taq* DNA polymerase. The reaction mixture was heated to 72°C for 5 min and cooled, and 300 ng of primer 2 was added prior to amplification by PCR, using 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 2 min, and extension at 72°C for 3 min; on the final cycle, the extension step was extended to 7 min. The PCR material was also purified by silica binding (Geneclean II; Bio 101) and repaired with T4 DNA polymerase. The material was then repurified by Geneclean II prior to phosphorylation with T4 polynucleotide kinase.

This material was confirmed as group C rotavirus cDNA by cross-hybridization to all group C dsRNA segments in Northern (RNA) blot analysis (Fig. 3). A schematic summarizing the strategy for synthesis of rotavirus cDNA from purified dsRNA is shown in Fig. 4.

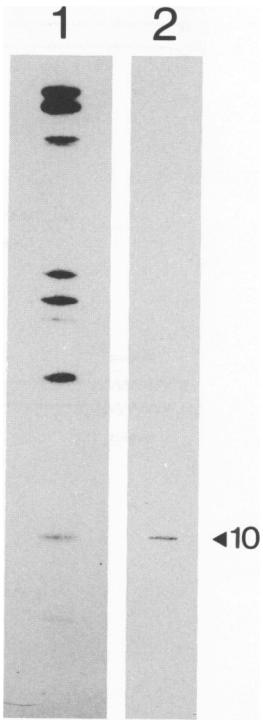

FIG. 3. Northern blot analysis of cDNA. Human (Bristol) group C rotavirus dsRNA was probed with ^{32}P-labelled cDNA products representing the complete group C rotavirus genome generated as described in the text (lane 1) and a ^{32}P-labelled PCR-generated (primer 2) full-length insert of gene 10 (lane 2).

Sequence analysis of genome segment 10. These PCR products were ligated with T4 DNA ligase into dephosphorylated *Sma*I-cleaved M13 mp8 and transformed into *Escherichia coli* JM101 by standard procedures (23). Two hundred recombinant plaques were obtained, and cloned genome segments were identified first by the size of the inserts (PCR with primer 2) and second by hybridization to dsRNA in Northern blots as previously described (11). These analyses together with preliminary sequence data confirmed that all recombinants were rotaviral in origin.

M13 recombinants of opposite polarity representing genome segment 10 (Fig. 3) were selected for detailed analysis. Nucleotide sequencing revealed the segment to be 728 bp long. Data base searching using the FASTA program (W. R. Pearson, Charlottesville, Va.) revealed an unexpected but limited homology (48%) with segment 11 of the group A rotaviruses; interestingly, there was no significant homology to group A segment 10. These findings suggested that the order of migration of segments 10 and 11 in the Bristol group C rotavirus is reversed relative to that in most group A rotaviruses (12). Segment 10 contained a single open reading frame of 636 bp coding for a protein of 212 amino acids (aa) with a predicted size of 23.7 kDa.

Data base analysis and direct alignment of the putative gene 10 protein revealed a limited homology of 38% in the C-terminal 39 aa with the human group A (Wa) gene 11, protein NS26 (16). Overall homology with group A NS26

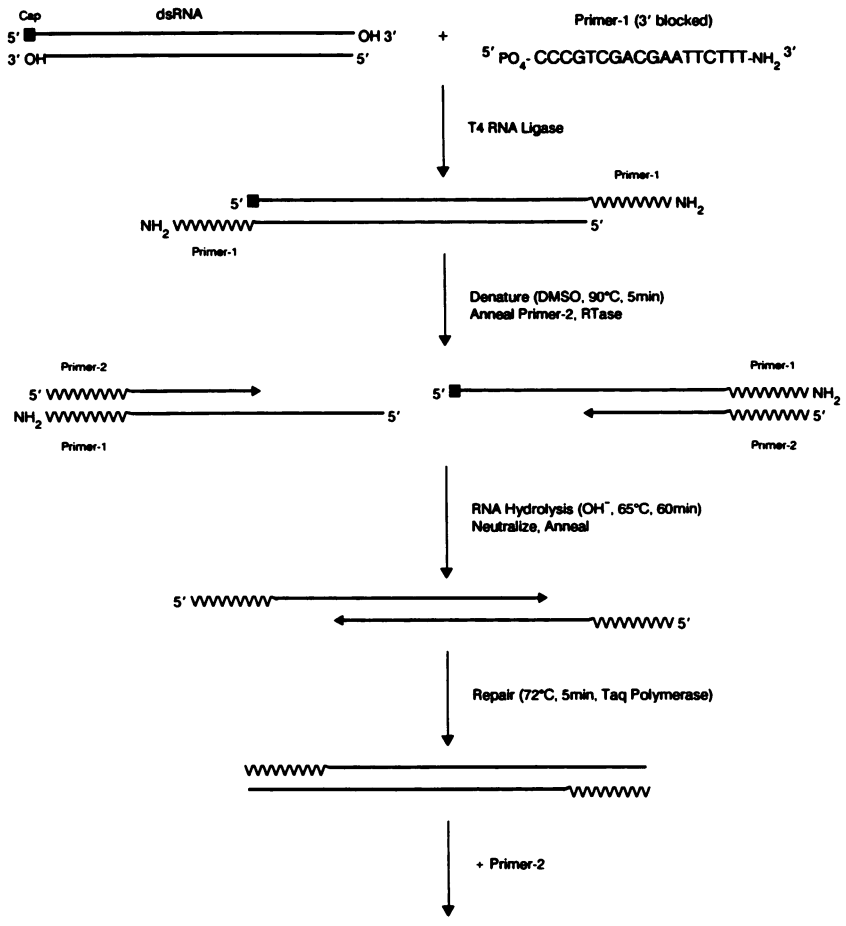

FIG. 4. Schematic of strategy for synthesis of cDNA from dsRNA segments. DMSO, dimethyl sulfoxide; RTase, reverse transcriptase.

was 16%, with an even lower identity of 12% with the group B (ADRV) rotavirus protein homolog (8). On the basis of the limited protein and nucleotide sequence homology with group A segment 11 but lack of significant homology with other rotavirus proteins, we would tentatively assign the group C genome segment 10 as coding for the equivalent of NS26.

The 5′ nucleotide sequence revealed features which closely mirrored the 5′ sequences of the group A viruses in that segment 10 began with GGC followed by an A/T-rich tract; this structure has been seen at the 5′ terminus of all other recombinants so far sequenced. The segment 10 gene was 728 bp long, and the open reading frame of 636 bp was preceded by a 5′ 19-bp untranslated region and followed by a 73-bp untranslated downstream sequence (Fig. 5). The putative methionine start codon at position 20 (ACAATGT) was not consistent with the strong initiation sequence (ANN ATGG) (17), whereas a second in-frame ATG at position 125 (AGAATGG) conforms more closely to the above Kozak consensus sequence.

The putative group C NS26 protein with a predicted length of 212 aa for the largest open reading frame was larger than both the group A (197 aa) and group B (170 aa) proteins. The group C protein was relatively rich in serine and threonine (16%), as seen for the group A (23%) and group B (16%) proteins. Unlike the human group A (Wa) and group B (ADRV) proteins, the group C segment 10 protein contained a single tryptophan residue. A total of four potential N-linked glycosylation sites (Asn-X-Ser/Thr) were present in the group C sequence (Fig. 5), contrasting with the two sites found in the groups A and B viruses.

Amplification and cloning strategy. Several similar methods involving polyadenylation of genomic dsRNA have been developed to facilitate the molecular cloning of dsRNA virus genomes (3, 16, 19). However, these have all relied on the availability of large quantities of starting material from which genomic dsRNA can be directly extracted. Alternative approaches to cloning have depended on purified rotaviruses to act as transcriptase factories generating large amounts of single-stranded RNA for use as a template in conventional cDNA synthesis (20). By using these approaches, the nucleotide sequences for many genome segments from different isolates, serotypes, and groups of rotaviruses have been determined.

Despite their initial discovery some 8 years ago, sequence information for atypical or non-group A human rotaviruses is limited, with only three dsRNA segments available for the group B human ADRV (8–10). The procedure necessitated purification of group B rotavirus from stool samples prior to analysis. Further, there were only two sequences available for human group C rotavirus genome segments (11, 20). The human (Bristol) group C rotavirus group antigen gene (VP6) was cloned (11) by reverse transcription and PCR, using primers designed from the Cowden strain of porcine group C

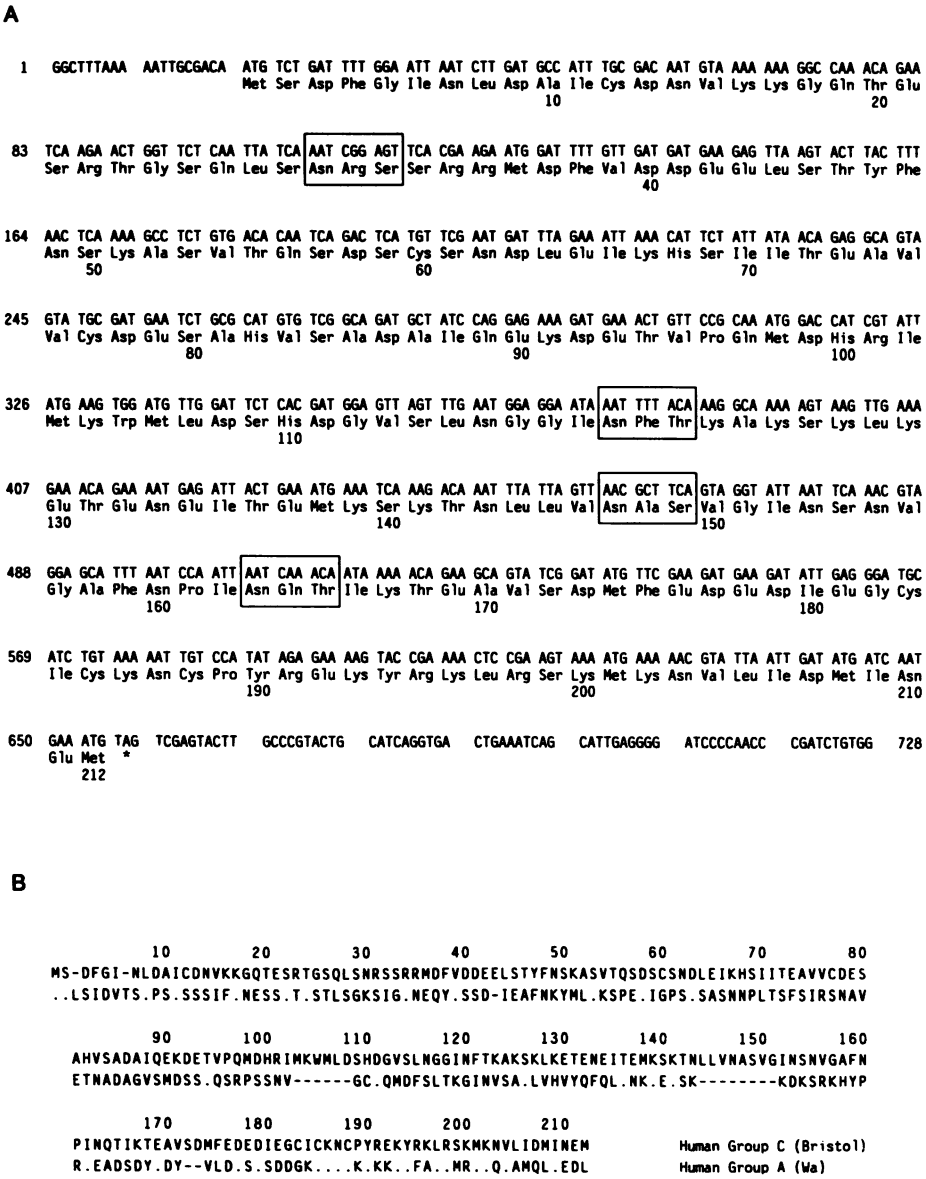

FIG. 5. (A) Nucleotide sequence and deduced amino acid sequence of the cDNA corresponding to human group C rotavirus genome segment 10. Potential N-linked glycosylation sites are shown as boxed areas. The methionine start codon is at nucleotide position 20, and the termination codon is at position 656. (B) Alignment of amino acid sequences of the human group C genome segment 10 putative protein (upper) with human group A (Wa) genome segment 11 NS26 protein (lower). Identical amino acids are represented as dots; gaps in the sequence alignment are shown as dashes. Alignments were performed by using the FASTA package (W. R. Pearson, Charlottesville, Va.).

rotavirus (4). Likewise, the production of full-length cDNA of the human group C rotavirus (strain 88-220) VP7-equivalent gene depended on the availability of nucleotide sequence from the Cowden strain of porcine group C rotavirus (20).

Earlier methods of cloning rotavirus dsRNA by tailing with poly(A) polymerase followed by oligo(dT)-primed reverse transcription required from 10 to 30 μg of dsRNA as starting material (3, 16, 19). These methods necessitated large preparations of gradient-purified rotaviruses grown in tissue culture. The method described here generated full-length cDNA from as little as 10 ng of each dsRNA genome segment.

The novel modifications presented in this report involved replacing the polyadenylation step with direct ligation of an oligonucleotide primer to the 3′ termini of the genome. By using an amino-linked modified oligonucleotide, concatenation was prevented, thus ensuring that only one primer was added to either end of each genome segment. Incorporation of a PCR step based on a complementary primer allowed amplification of any dsRNA virus genome without prior knowledge of the sequence. Often the limited amount of material in rotavirus clinical samples will allow only preliminary identification by electropherotyping, enabling a basic differentiation of isolates into group A or non-group A. The technique described provides a general method for amplifying dsRNAs from clinical samples. This method should allow detailed characterization of dsRNA from the many and

diverse noncultivatable viruses using this form of genetic material without the need for virion purification or prior knowledge of nucleotide sequence information.

Nucleotide sequence accession number. The complete nucleotide sequence of genome segment 10 is available from GenBank under accession number M81488.

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